International Journal of Pharmaceutics 413 (2011) 167–173

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

A novel bottom-up process to produce nanoparticles containing protein and peptide for suspension in hydrofluoroalkane propellants

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ARTICLE INFO

Article history:
Received 6 January 2011
Received in revised form 28 March 2011
Accepted 31 March 2011
Available online 16 April 2011

Keywords:
Tert-butyl alcohol/water co-solvent
Freeze-drying
Nanoparticle
Hydrofluoroalkane
Pressurized metered dose inhaler
Protein and peptide

ABSTRACT

To overcome the disadvantages of microemulsion and nanoprecipitation methods to produce protein-containing nanoparticles, a novel bottom-up process was developed to produce nanoparticles containing the model protein lysozyme. The nanoparticles were generated by freeze-drying a solution of lysozyme, lecithin and lactose in tert-butyl alcohol (TBA)/water co-solvent system and washing off excess lecithin in lyophilizate by centrifugation. Formulation parameters such as lecithin concentration in organic phase, water content in TBA/water co-solvent, and lactose concentration in water were optimized so as to obtain desired nanoparticles with retention of the bioactivity of lysozyme. Based on the results, 24.0% (w/v) of lecithin, 37.5% (v/v) of water content, and 0.56% (w/v) of lactose concentration were selected to generate spherical nanoparticles with approximately 200 nm in mean size, 0.1 in polydispersity index (PI), and 99% retained bioactivity of lysozyme. These nanoparticles rinsed with ethanol containing dipalmitoylphosphatidylcholine (DPPC), Span 85 or oleic acid (3%, w/v) could readily be dispersed in HFA 134a to form a stable suspension with good redispersibility and 98% retained bioactivity of lysozyme. The study indicates there is a potential to produce pressed metered dose inhaler (pMDI) formulations containing therapeutic protein and peptide nanoparticles.

1. Introduction

Pulmonary administration became an attractive delivery route for systemically acting proteins and peptides (Li and Seville, 2010) to enhance patient compliance for biodrugs (Antosova et al., 2009). Pressed metered dose inhaler (pMDI) was used as delivery devices for proteins and peptides by many research groups (Bailey and Berkland, 2008) and was well accepted by both patients and clinicians due to its good compliant such as noninvasiveness, portability and disposability (Shoyele and Slowey, 2006; Terzano, 2001).

Most drugs, including proteins and peptides, exhibit negligible solubility in hydrofluoroalkane (HFA) propellants used in the preparation of pMDI systems. Therefore, suspension formulations employing micronized drug powders are required (Li and Seville, 2010). Currently, inhalation therapy using drug-containing nanoparticles has been recommended as an alternative to conventional micronized particles or large porous microparticles, since nanoparticles can be deposited in the lung and escape through both phagocytic and mucociliary clearance mechanisms (Tsapis et al., 2002). Nanoparticles possess an excellent geometric size that could positively influence the aerodynamic diameter of a pulmonary drug delivery system so that pMDI formulation containing drug nanoparticles could improve the fraction of fine particle drug.

The combination of tert-butyl alcohol (TBA) and water is one of the most extensively evaluated non-aqueous co-solvent systems, which has been used in the manufacture of marketed injectable pharmaceutical products, such as CAVERJECT® Sterile Powder (Teagarden and Baker, 2002). The low toxic TBA (Qian et al., 2008) freezes completely in most commercial freeze-dryers, readily sublimes during primary drying (Kasraian and DeLuca, 1995a) due to its high freezing, eutectic temperatures and high vapor pressure (Kasraian and DeLuca, 1995b). Therefore, lyophilization efficiency can be greatly enhanced with the use of TBA/water co-solvent (Wang et al., 2010). Freeze-drying of TBA/water co-solvent system had been used to produce nanocrystals of a poorly water-soluble drug to enhance its solubility, and further to improve its bioavailability (De Waard et al., 2008; Purvis et al., 2007). It should be

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doi:10.1016/j.ijpharm.2011.03.069

pointed out that freeze-drying of TBA/water co-solvent system in the absence of surfactant might be unsuitable for proteins to fabricate nanoparticles because proteins are known to be surface-active. Zhang et al. (2009) had reported that insulin could survive the lyophilization process in TBA/water co-solvent system with the presence of surfactant and sugar, and the structural stability and activity of insulin were well retained in the process. Due to its low phase-change temperatures, lecithin may be added in TBA/water co-solvent as a surfactant to protect conformation of protein during lyophilization.

Usually drug nanoparticles for pulmonary drug delivery are created by milling down larger particles (top-down process) or precipitating out of liquid phase (bottom-up process) (Rabinow, 2004). Recently, Nyambura et al. (2009b) reported that nanoparticles containing a model protein lysozyme had been produced using microemulsion or nanoprecipitation methods coupled with freeze-drying so that they could be readily dispersed in HFA 134a propellant of pMDI. However, for both microemulsion and nanoprecipitation, the processes were relatively complex and the liquid containing protein was homogenized to reduce the size of emulsion droplets by high-speed shearing force, which might destroy the molecular structure of protein. Furthermore, because of the use of organic solvents with low freezing points such as chloroform, dichloromethane and ethanol during emulsification, the freeze-drying of emulsion containing protein at −110 °C to −115 °C (Nyambura et al., 2009a,b) consumed much more electric energy than freezing-drying at conventional temperature (−50 °C to −55 °C). This does not conform to the policy of saving energy and reducing emission into environment.

To overcome these disadvantages of microemulsion and nanoprecipitation methods to produce protein nanoparticles and simplify the process, a novel bottom-up process was developed in present study to fabricate nanoparticles containing a bioactive model protein, lysozyme, for pMDI formulation. Nanoparticles containing lysozyme were produced by dissolving lysozyme in TBA/water co-solvent system in the presence of lecithin as surfactant and lactose as cryoprotectant, followed by a freeze-drying and purification process. Formulation factors were optimized in order to produce nanoparticles with desired characteristics (i.e. size and retained bioactivity of lysozyme). Furthermore, the dispersibility of the nanoparticles in HFA 134a and retention of lysozyme's bioactivity in HFA 134a suspension were evaluated. To the best of authors' knowledge, for the first time, the TBA/water co-solvent system in the presence of surfactant and cryoprotectant was used to produce protein and peptide nanoparticles, which can be well dispersed in HFA 134a.

2. Materials and methods

2.1. Materials

Tert-butyl alcohol, isooctane, absolute ethanol, sodium hydroxide, monopotassium phosphate, and phosphotungstic acid were obtained from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China) while HFA 134a was purchased from INEOS Flour Ltd. (Runcorn, Cheshire WA74JE, UK). Lactose, oleic acid, Span 85, and dipalmitoylphosphatidylcholine (DPPC) were obtained from Sigma–Aldrich (Shanghai) Trading Co. Ltd. (Shanghai, China). Lecithin (Lipoid E80), lysozyme and *Micrococcus Lysodeikticus* were purchased from Lipoid GmbH (Ludwigshafen, Germany), AMRESCO Inc. (Ohio, USA) and Shanghai Touching Tech. Co., Ltd. (Shanghai, China), respectively. Water was distilled via a super pure water system (PureLAB Option, ELGA Lab Water Inc., UK).

2.2. Methods

2.2.1. Preparation of lysozyme-containing nanoparticles

25 mg of lysozyme and 10 mg of lactose were dissolved in 1.8 mL of super purified water as the aqueous phase. Lecithin was weighted into separate vials and dissolved in 3.0 mL of TBA to form the organic phase at 26–30 °C. Lecithin concentration in the organic phase was varied from 8% to 40% (w/v) (i.e. 240, 480, 720, 960 and 1200 mg, respectively) to investigate the optimal concentration for production of nanoparticles. The aqueous phase was then added into the organic phase under vortex or stirring to form a pale yellow clear solution at 26–30 °C. Immediately the solution was snap frozen by immersing in liquid nitrogen and then lyophilized using a CHRIST ALPHA 1-4LSC freeze dryer (Osterode, Germany) for a minimum of 12 h at −55 °C under 0.25 mbar to remove water and TBA. This resulted in lyophilizate containing nanoparticles covered with surfactant. Then the nanoparticles were suspended in absolute ethanol, in which lecithin was freely soluble while lysozyme and lactose were insoluble, and thus the structure of nanoparticles could be preserved. The nanoparticles in the raw suspension were separated from free surfactant by centrifugation using TGL-16C desk centrifuge (Feige Brand, Shanghai Anting Scientific Instruments Factory, Shanghai, China) with the centrifuge speed of 15,000 rpm (equivalent to approximately 32,000 × *g*) at 25 °C. Polytetrafluoroethylene centrifuge tubes (5 mL, Shanghai ChuBai Laboratory Equipment Co., Ltd., China) were used due to their excellent solvent compatibility and ease of nanoparticles collection from the non-stick surface. The solvent containing surfactant was dumped and the sediments containing nanoparticles were collected for centrifuging repeatedly to ensure purification of the nanoparticles (Cook et al., 2005).

In order to investigate the effect of (a) water content in TBA/water co-solvent and (b) lactose concentration in aqueous phase on nanoparticles containing lysozyme, the preparation of the aqueous phase was described as follows: (a) 25 mg of lysozyme and 10 mg of lactose were dissolved in different amount of super purified water (i.e. 1.6, 1.8, 2.0, 2.2, and 2.4 mL), or (b) 25 mg of lysozyme and different amount of lactose (i.e. 0, 5, 10, 15, and 20 mg) were dissolved in 1.8 mL of super purified water. In both cases, 700 mg of lecithin was dissolved in 3.0 mL of TBA to form the organic phase at 26–30 °C. The rest of procedures was the same as described above.

2.2.2. Assay for retained bioactivity of lysozyme

Bioactivity of lysozyme was assayed by modifying the method reported by Nyambura et al. (2009b). Appropriate weight of samples (equivalent to 5 mg of lysozyme) or 5 mg of control lysozyme was weighed in triplicate into vials. Into each vial, 5 mL of phosphate buffer solution (pH 7) was added and shaken well to form a clear solution. Then, 200 μL of each solution was measured into a clean vial and diluted to 10.0 mL with buffer solution. Substrate suspension was prepared by adding a small amount of buffer solution in 3 mg of dry powder of *Micrococcus Lysodeikticus* and grinding in a mortar for 2 min and then diluting to 25 mL with buffer solution.

Double beam UV/vis spectrophotometer (Model Tu-1901, Beijing Puxi General Instrument Co., Ltd., China) was set at wavelength of 450 nm and 25 °C for measurement. Firstly, 2.5 mL of substrate suspension was pipetted into 1 cm quartz cuvettes following incubation for 5 min to achieve temperature equilibration. Then 100 μL of each sample or control solution was added to the test cuvette, and the solutions were mixed immediately by inversion. The decrease in $A_{450\,nm}$ was recorded for 10 min. The same procedure was followed for a blank rate except that 100 μL of sample or control solution was replaced by the same volume of buffer solution. The changes in absorbance units of samples, controls and blank were used to calculate the percent-retained activity of each sample.

2.2.3. Particle size analysis for nanoparticles

The size of nanoparticles was measured via Photon Correlation Spectroscopy (PCS) (Malvern Zetasizer Nano ZS90, Malvern Instruments, Malvern, UK). Hydrodynamic diameter, expressed as *Z*-average diameter, was measured and polydispersity index (PI) indicating the width of particle size distribution was determined by cumulant analysis as described in the International Standard on PCS. At first, nanoparticles were homogeneously dispersed in isooctane, which was initially filtered through a 0.1 μm nylon membrane filter (Tengjin®, China), by sonication (Ningbo Xinzhi Biotechnology Co., Ltd., Zhejiang, China) in water bath for 5 min. Then the sample concentration was adjusted to 6 mg of nanoparticles/mL of isooctane, which was adequate to provide required analytical count rate in the PCS (>50 kilocounts per second). Subsequently, the suspension was transferred into a non-frosted quartz cuvette, placed in the sample holder of PCS, and stood for 2 min to reach equilibrium prior to measurement. Each sample was measured in triplicate.

2.2.4. Transmission electron microscopy (TEM) for nanoparticles

Lysozyme-containing nanoparticles produced with the optimized formulation, suspended in isooctane, were placed on the copper grids to allow isooctane to evaporate and then the nanoparticles were treated by negative staining with phosphotungstic acid (PTA) solution (1%, w/v) and dried under conditions of less than 45% relative humidity at ambient temperature before loaded in the microscope. The prepared samples were observed by JEM-1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan).

2.2.5. Dispersion characteristics of nanoparticles in HFA 134a

Surfactants such as DPPC, oleic acid and Span 85 are commonly used as dispersing stabilizers in pMDIs. In order to investigate the dispersibility of lysozyme-containing nanoparticles in HFA 134a propellant, four groups of nanoparticles were prepared and purified following the procedure described above. The four groups of nanoparticles were separately dispersed in 4.5 mL of solvent including ethanol, 3% (w/v) oleic acid in ethanol, 3% (w/v) Span 85 in ethanol, and 3% (w/v) DPPC in ethanol, and then separated from the medium by centrifugation as the method reported by Nyambura et al. (2009b). Then 35 mg of lyophilizate (nanoparticles) from each group was placed into a plastic coated glass bottle (Shandong Jewim Pharmaceutical Co., Ltd., Shangdong, China). 8 g of propellant HFA 134a was filled through the valve using an aerosol filling machine (Zhongshan Zhihua Aerosol Equipment Co., Ltd., Guangdong, China) after a 50 μL valve (Valois (Suzhou) Dispensing Systems Co., Ltd., China) was immediately crimped onto the bottle using a semi-automatic bottle crimper (Zhongshan Zhihua Aerosol Equipment Co., Ltd., Guangdong, China). The pMDI formulations were then sonicated (Ningbo Xinzhi Biotechnology Co., Ltd., Zhejiang, China) in water bath for approximately 2 min and the suspension stability was visually checked for any obvious separation, flocculation, coalescence or sedimentation of the dispersed nanoparticles as time went on. The redispersibility of the nanoparticles in HFA 134a was visually evaluated via shaking the formulation by hand to imitate patient use. This operation was repeated twice.

2.2.6. Retention of bioactivity of lysozyme in HFA 134a

Proteins may be physically unstable due to gravity setting when stored in a hydrophobic environment (Williams and Liu, 1999). In order to determine whether the activity of lysozyme was retained after lysozyme-containing nanoparticles were suspended in HFA 134a, three batches of nanoparticles were produced and the bioactivity of lysozyme-containing nanoparticles was determined as the method reported by Nyambura et al. (2009b). Each batch of nanoparticles and HFA 134a were formulated into pMDI suspensions and were left to stand overnight at room temperature before analysis.

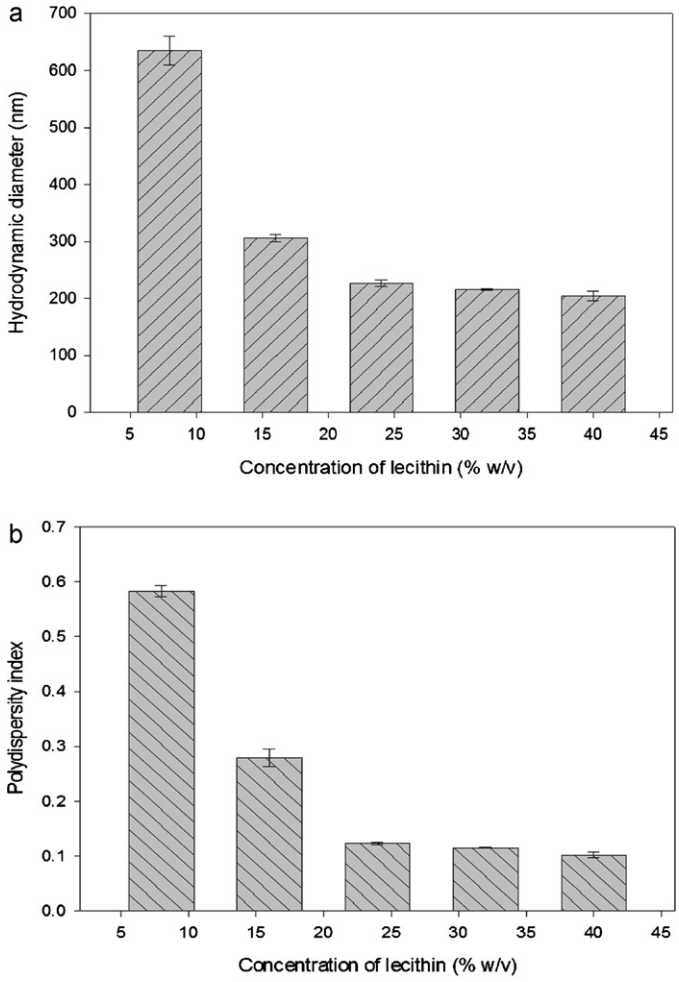

Fig. 1. Effect of lecithin concentration on (a) the particle size and (b) polydispersity index (PI) of lysozyme-containing nanoparticles. Each point is the mean (±S.D.) of 3 preparations.

The pMDI suspensions were immersed in liquid nitrogen to chill the propellant. Once cold, the bottles were removed from liquid nitrogen and the valves were removed to allow HFA 134a to volatilize slowly, leaving the nanoparticles at the bottom of the bottles. The activity of lysozyme retained in the nanoparticles was determined as the method described previously.

2.2.7. Statistical analysis

All data were presented as mean ± S.D. and analyzed using Student's *t*-test or one-way ANOVA. Statistical significance was determined at a value $p < 0.05$.

3. Results

3.1. Effect of lecithin concentration on the size of lysozyme nanoparticles

Nanoparticles containing lysozyme were produced using TBA/water co-solvent system in the presence of surfactant and cryoprotectant. The concentration of surfactant lecithin was optimized in order to produce nanoparticles in the desired size range. The average hydrodynamic diameter of the produced nanoparticles was in the range of 204–635 nm (Fig. 1a) with the values of PI ranged 0.1–0.6 (Fig. 1b). As lecithin concentration increased in the organic phase, the average particle size and PI values were reduced until the lecithin concentration reached 24.0% (w/v). Further increase of

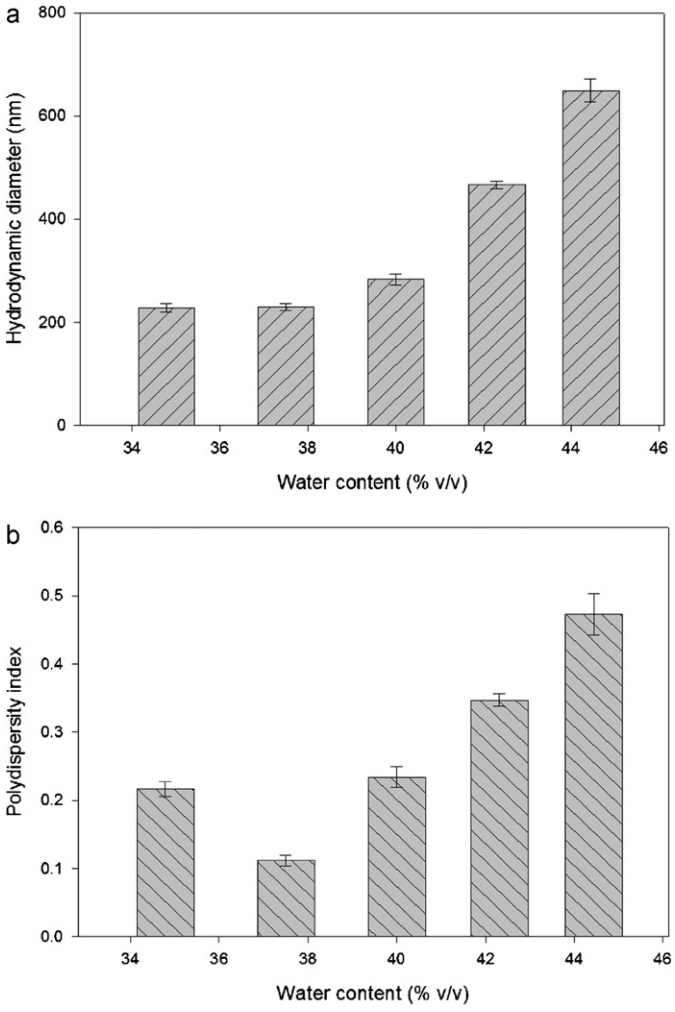

Fig. 2. Effect of water content in TBA/water co-solvent system on (a) the particle size and (b) PI of lysozyme-containing nanoparticles. Each point is the mean (±S.D.) of 3 preparations.

lecithin concentration had minor effect on the nanoparticle size and PI. It is important to note that higher concentration of lecithin led to more surfactant covering the surface of nanoparticles and thus reducing the mean particle size and PI. However, the surfactant also affected the product purification and recovery process as it increased viscosity of the suspension, which hampered the sedimentation of nanoparticles during centrifugation. Thus, optimal concentration of lecithin was chosen to ensure manufacture of nanoparticles with desirable particle size and proper viscosity for processing. Based on the results, a preparation containing 24.0% (w/v) lecithin in organic phase was selected for subsequent investigations.

3.2. Effect of water content on the size of lysozyme nanoparticles

The water content in TBA/water co-solvent system was also optimized in order to produce nanoparticles in the desired size range. The results in Fig. 2a showed the average hydrodynamic diameter of nanoparticles increased from 228.0 ± 8.2 nm to 642.3 ± 13.7 nm with water content increasing from 34.8% (v/v) to 44.4% (v/v). The mean size of nanoparticles slightly increased as water content in the range of 34.8–40.0% (v/v), but rapidly increased when water content was above 40.0% (v/v). Values of PI were in the range of 0.13–0.50 with a minimum of 0.11 corresponding to 37.5% (v/v) of water content as shown in Fig. 2b. When water content was below 33.3% (v/v), lysozyme and lactose could not be fully dissolved in TBA/water co-solvent to form a single-phase solution (data not shown). Therefore, a preparation containing 37.5% (v/v) water in TBA/water co-solvent system was selected for subsequent investigations.

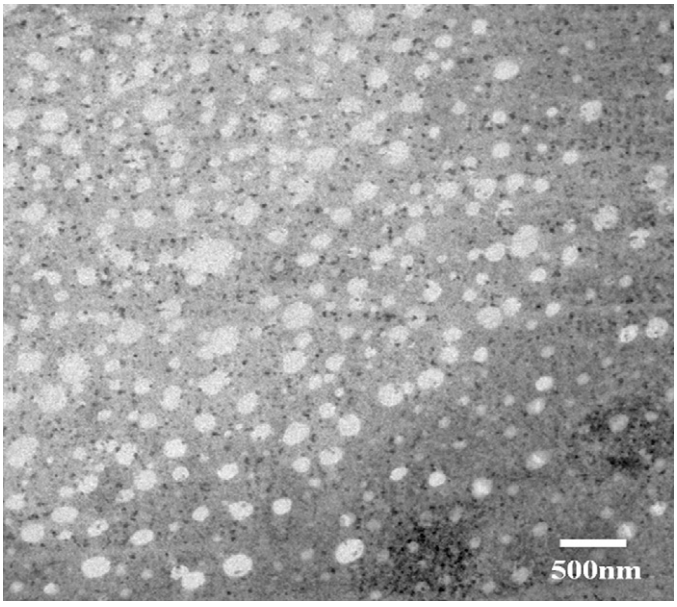

Fig. 3. TEM micrograph of lysozyme-containing nanoparticles (light dots represent the nanoparticles).

3.3. Effect of lactose concentration on the size of lysozyme nanoparticles and retained bioactivity of lysozyme

Lactose approved to use in dry power inhalation by FDA (1998) was used in TBA/water co-solvent system as cryoprotectant and lyoprotectant to retain the activity of lysozyme. The effect of its concentration on the size of nanoparticles and retained bioactivity of lysozyme was investigated. The results in Table 1 showed lactose concentration barely had impact on the hydrodynamic diameter and PI of lysozyme nanoparticles (one-way ANOVA, $p > 0.05$), but had significant effect on retained biological activity of lysozyme (one-way ANOVA, $p < 0.05$). Approximately 99% (relative to unprocessed lysozyme raw material) bioactivity of lysozyme was retained in nanoparticles when no less than 0.56% (w/v) lactose was used in aqueous phase. Therefore, 0.56% (w/v) lactose (i.e. the lactose–lysozyme mass ratio of 0.4) was selected as cryoprotectant and lyoprotectant to retain the bioactivity of lysozyme.

3.4. Morphology of lysozyme-containing nanoparticles

TEM micrograph of the nanoparticles (Fig. 3) showed that the lysozyme-containing nanoparticles were approximately in spherical shape with about 200 nm in mean size. Typical size distribution curve of the nanoparticles measured via Malvern Zetasizer Nano ZS90 was shown in Fig. 4. The particle size and size distribution of lysozyme-containing nanoparticles determined from TEM micrograph are in agreement with Malvern curve measured via PCS.

3.5. Dispersion of nanoparticles in HFA 134a

Good redispersibility of the creamed formulation after shaking is important for dose reproducibility of suspension pMDIs. The results of dispersibility study showed that the addition of surfactant in the final rinsing solution resulted in suspension of nanoparticles

Table 1
Effect of lactose concentration on characteristics of lysozyme-containing nanoparticles. Each result is the mean (±S.D.) of 3 preparations. 25 mg of lysozyme was in the original formulation.

Lactose (mg)	Lactose concentration (%, w/v)	Hydrodynamic diameter (nm)	Polydispersity index	Retained activity of lysozyme (%)
0	0	211.3 ± 9.39	0.130 ± 0.012	55.3 ± 2.8
5	0.28	217.3 ± 8.22	0.125 ± 0.012	81.2 ± 2.4
10	0.56	220.0 ± 9.09	0.114 ± 0.011	99.9 ± 4.0
15	0.83	224.3 ± 9.39	0.133 ± 0.018	99.8 ± 3.0
20	1.11	228.3 ± 11.0	0.138 ± 0.012	99.8 ± 2.4

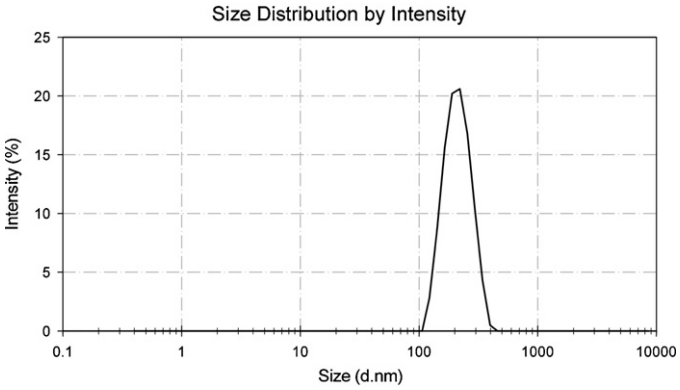

Fig. 4. Typical size distribution curve of lysozyme-containing nanoparticles (Z_{ave} = 218 nm; PI = 0.12).

Table 2
Effect of surfactant on suspension stability of nanoparticles in HFA 134a propellant.

Final rinsing solution	Suspension stability for 1 min
Ethanol	Unstable
3% (w/v) DPPC in ethanol	Stable
3% (w/v) oleic acid in ethanol	Stable
3% (w/v) Span 85 in ethanol	Stable

in HFA 134a stable for at least 1 min (Table 2). It was observed that a homodisperse suspension was formed immediately after hand-shaking and maintained stability for at least 1 min when the nanoparticles were rinsed with ethanol containing surfactant such as DPPC, oleic acid or Span 85. Moreover, the 1-min stability can ensure the patient to dispense the formulation uniformly to inhalation after hand-shaking the suspension pMDI. However, when the nanoparticles were rinsed with absolute ethanol, agglomeration followed by creaming occurred immediately after hand-shaking the suspension containing nanoparticles. Therefore, addition of surfactant in rinsing ethanol was helpful to improve the stability of suspension of nanoparticles in HFA 134a.

3.6. Retention of bioactivity of lysozyme in HFA 134a

Based on the statistical analysis of measured activity of lysozyme, it was found there was no significant difference (*t*-test, $p > 0.05$) between the retained bioactivities of lysozyme before (99 ± 4%) and after (98 ± 3%) the lysozyme-containing nanoparticles were suspended in HFA 134a ($n = 3 \pm$ S.D.). The results are in agreement with previous studies where the retention of bioactivity of proteins in HFA pMDIs was proved (Liao et al., 2005; Nyambura et al., 2009b; Quinn et al., 1999).

4. Discussion

In this study, a novel process was developed to prepare nanoparticles containing protein and peptide. These nanoparticles were prepared by freeze-drying a single-phase solution of protein, a sugar and surfactant in TBA/water co-solvent system and washing off excess lecithin in lyophilizate. The formulation was investigated and the results indicated that surfactant lecithin concentration in organic phase and water content in TBA/water co-solvent had significant effect on the size and polydispersity index (PI) of nanoparticles containing model protein lysozyme. An optimal formulation containing 24.0% (w/v) lecithin in organic phase, 37.5% (v/v) water in TBA/water co-solvent and 0.56% (w/v) lactose in water was determined to produce lysozyme-containing nanoparticles with approximately 200 nm in mean size and 0.1 in the polydispersity index (PI). Lactose concentration in aqueous phase had significant effect on the retained bioactivity of lysozyme, but barely affected the hydrodynamic diameter and PI of lysozyme nanoparticles. The results demonstrated that about 99% activity of lysozyme relative to the unprocessed raw material could be retained in nanoparticles when 0.56% (w/v) lactose was added in aqueous phase. The formulation with a high concentration of lactose and the production process of the nanoparticles in this study ensured the stability of lysozyme, while a minimum mass ratio of 0.25 for lactose–enzyme was previously reported (Nyambura et al., 2009b). Moreover, the use of lecithin may maintain the secondary and tertiary structures of lysozyme and protect lysozyme from degradation during processing because lecithin is more surface-active than lysozyme.

The surface-active protein lysozyme might alter the size of droplets and lead to increase of nanoparticles' size during freeze-drying. However, when a large amount of lecithin was added, protein molecules would be displaced from the interface of water droplet-crystal of TBA hydrate by lecithin during freeze-drying, and thus the droplets with decreased size would result in nanoparticles with desired size and size distribution. By using TBA/water co-solvent as lyophilization solvent, particles in nanoscale size were produced in current study and this is in agreement with the study conducted by De Waard et al. (2008). However, smaller particles with narrower size distribution were obtained in current study. This is very likely because a large amount of lecithin was used to coat the nanoparticles, provided steric hindrance between the particles, and thus reduced their contact chances. The results demonstrated that the mean size and PI of the nanoparticles decreased with the amount of lecithin increasing in organic phase. This indicated that the mechanism of nanoparticle formation via this novel process using a large amount of lecithin might be different from that previously reported (Nyambura et al., 2009a,b).

As one of the bottom-up processes to produce nanoparticles, the mechanism of nanoparticle formation via the novel process may be different from those of emulsification and nanoprecipitation methods (Nyambura et al., 2009b). Microemulsion was taken as templates in emulsification method (Anton et al., 2008) and a great deal of non-solvent was added into drug solution in nanoprecipitation method (Bilati et al., 2005). It is well known that there are two steps of nuclei forming and their growth when crystals or precipitates are separated from a solution containing compounds. Snap freeze and surfactants are always applied since the freezing rate and surfactant molecules could control the size of nucleus or precipitate (De Waard et al., 2008; Dixit et al., 1998; Lai et al., 2005; Liao and Liao, 2007; Liu et al., 2004; Pileni, 2003). In the freezing process of solution of protein, lactose and lecithin in the TBA/water

co-solvent system, phosphatidylcholine (main ingredient of lecithin) molecules prior adsorbed in the surface of TBA hydrate crystals to reduce the interfacial tension because of the new interface generation. In the process of absorption, phosphatidylcholine molecules might have formed a special arrangement, which the head group of the phosphatidylcholine molecule might be orientated to the unfrozen solution of protein and lactose while hydrocarbon chain part might be orientated towards inside the TBA hydrates crystal. In addition, it is also important to snap-freeze TBA/water co-solvent system using liquid nitrogen since faster freezing may result in higher TBA hydrate and solute (i.e. lysozyme and lactose) nucleation rate. Therefore, more nuclei are formed to grow up in more crystals, and consequently formed more particles in smaller size. Finally, such single-phase solution frozen might be equivalent to "solid microemulsion" which the droplet of water solution containing protein and lactose, lecithin and TBA hydrate is an internal phase, a surfactant and a continuous phase, respectively. Lecithin plays an important role to coat on the surface of internal phase during freeze-drying and provide steric hindrance to reduce the contacts of nanoparticles containing lysozyme in lyophilizate.

For suspension pMDIs, it is important that drug is insoluble in suspending medium to retain the particle structure and the particle size is relatively stable during storage. In this study, surfactant in co-solvent system containing ethanol was necessary to stabilize lysozyme-containing nanoparticles in HFA 134a. With the presence of surfactant at the interface of nanoparticles-HFA, suspension pMDI could keep well redispersibility on storage as it became creaming after hand shaking and the dispersion was physically stable for at least 1 min. Moreover, with the presence of surfactant the bioactivity of lysozyme was well retained after lysozyme-containing nanoparticles were suspended in HFA 134a, and this is in agreement with the work of Nyambura et al. (2009b). It was reported when proteins were stabilized by sucrose or trehalose and/or 80% hydrolyzed polyvinyl alcohol, the retention of biological activity of lysozyme and catalase was achieved in pMDI formulations stored for up to 6 months at ambient temperature (Liao et al., 2005). Similarly, studies of lysozyme structures in both HFA 134a and HFA 227 using FT-Raman spectroscopy have shown retention of lysozyme structural stability (Quinn et al., 1999). The current study and these positive literature reports indicate that it is potential to develop suspension pMDIs containing stable macromolecules such as protein, peptide, gene and vaccine for aerosol drug delivery. Similarly, water-soluble drugs or hydrophilic drugs may also be produced into pure drug nanoparticles via the novel process for aerosol inhalation administration.

Lysozyme-containing nanoparticles fabricated by the novel process in HFA 134a pMDI formulation showed similar properties to those produced by nanoprecipitation previously reported by Nyambura et al. (2009b). However, compared to the previous methods, the novel process definitely presented the advantages of simplicity and high efficiency to produce lysozyme-containing nanoparticles. Firstly, the process of preparing nanoparticles containing protein was simplified and high-speed shearing force, which may potentially destroy the molecular structure of protein and peptide, was avoided. Secondly, the use of TBA/water co-solvent system markedly enhanced lyophilization efficiency and reduced energy consumption when the freeze-drying was carried out at conventional temperature ($-50\,^{\circ}C$ to $-55\,^{\circ}C$). In addition, lysozyme-containing nanoparticles fabricated by the novel process were in the size range more suitable for pulmonary delivery.

5. Conclusion

In conclusion, a novel process of using TBA/water co-solvent system in the presence of surfactant and cryoprotectant coupled with freeze-drying was developed to fabricate protein and peptide nanoparticles. It had the advantages of process simplicity and high efficiency compared to previously reported emulsification and nanoprecipitation methods. The nanoparticles fabricated from the optimized formulation showed suitable size and narrow size distribution for pulmonary delivery, and were readily dispersed in HFA 134a with the help of ethanol as co-solvent and addition of surfactant to stabilize the suspensions. Bioactivity of entrapped lysozyme in the nanoparticles was remained after preparation and dispersion in HFA 134a propellant. This indicated the potential of nanoparticles for delivery of protein and peptide from HFA-based pMDI formulations. The long-term stability and *in vitro* deposition efficiency of nanoparticles containing protein and peptide in pMDI formulations will be investigated in future studies.

Acknowledgements

The authors thank Madame Danlei Shen, a pMDI senior sale's manager, Valois (Suzhou) Dispensing Systems Co., Ltd., a subcompany of Aptar Pharma in China for kindly donating 50 μL metered valves used in this research.

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